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Regulated endosomal trafficking of Diacylglycerol lipase alpha (DAGLα) generates distinct cellular pools; implications for endocannabinoid signaling.

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Ya Zhou[1], Fiona V. Howell[1], Oleg O. Glebov[1], David Albrecht[2*], Gareth Williams[1], and Patrick Doherty[1]

[1]Wolfson Centre for Age-Related Diseases, King's College London, UK, SE1 1UL.

[2]Randall Division of Cell and Molecular Biophysics, King's College London, UK, SE1 1UL.

*Present address: MRC Laboratory for Molecular Cell Biology, University College London, UK, WC1E 6BT.

Corresponding authors: -
Professor Patrick Doherty
Wolfson Centre for Age-Related Diseases, King's College London, SE1 1UL
Tel 0207 848 6811
Fax 0207 848 6816
Email patrick.doherty@kcl.ac.uk

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Abstract

Diacylglycerol lipase alpha (DAGLα) generates the endocannabinoid (eCB) 2-arachidonylglycerol (2-AG) that regulates the proliferation and differentiation of neural stem cells and serves as a retrograde signaling lipid at synapses. Nothing is known about the dynamics of DAGLα expression in cells and this is important as it will govern where 2-AG can be made and released. We have developed a new construct to label DAGLα at the surface of live cells and follow its trafficking. In hippocampal neurons a cell surface pool of DAGLα co-localizes with Homer, a postsynaptic density marker. This surface pool of DAGLα is dynamic, undergoing endocytosis and recycling back to the postsynaptic membrane. A similar cycling is seen in COS-7 cells with the internalized DAGLα initially transported to EEA1 and Rab5-positive early endosomes via a clathrin-independent pathway before being transported back to the cell surface. The internalized DAGLα is present on reticular structures that co-localize with microtubules. Importantly, DAGLα cycling is a regulated process as inhibiting PKC results in a significant reduction in endocytosis. This is the first description of DAGLα cycling between the cell surface and an intracellular endosomal compartment in a manner that can regulate the level of the enzyme at the cell surface.

Introduction

The diacylglycerol lipases (DAGLα & DAGLβ) are the enzymes that synthesize 2-arachidonylglycerol (2-AG), an endogenous ligand for the cannabinoid receptors type 1 and 2 (CB1 and CB2). DAGL-dependent endocannabinoid (eCB) signaling regulates synapse formation during development by modulating axonal growth and guidance and, in the postnatal brain, regulates neural stem cell proliferation and neuroblast migration (Oudin et al., 2011a; Oudin et al., 2011b; Zhou et al., 2015). At developed synapses this pathway also regulates synaptic plasticity via retrograde signaling (Ohno-Shosaku and Kano, 2014). In the above context, DAGLα and the CB1 receptor co-localize in axonal growth cones in developing neurons but whereas the CB1 receptor remains expressed presynaptically at developed synapses, DAGLα becomes restricted to the postsynaptic dendritic spine (Bisogno et al., 2003; Uchigashima et al., 2007; Williams et al., 2003; Yoshida et al., 2006). This selective localization of DAGLα to dendritic spines in developed neurons highlights how the regulated compartmentalization of this enzyme can determine eCB function.

Knockout (KO) mice studies revealed that depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) are lost in hippocampus, cerebellum and striatum in DAGLα-/- mice (Gao et al., 2010; Tanimura et al., 2010). The modulation of synaptic strength by 2-AG regulates many behaviors including appetite, pain and cognition (Alger and Kim, 2011) and DAGLα and CB1 receptor KO mice share the same disrupted phenotypes (Powell et al., 2015). The adverse psychiatric consequences of inhibiting CB1 function in humans (Moreira and Crippa, 2009) are also likely to be consequential to the disruption of 2-AG signaling at synapses as DAGLα KO mice have been used to demonstrate a causative link between synaptic eCB signaling and anxiety and depressive behaviors (Shonesy et al., 2014). Thus, it is important to understand how DAGLα is trafficked in the dendrites and the cell body of neurons and other cells.

A ~370 amino acid C-terminal tail differentiates DAGLα from DAGLβ (Reisenberg et al., 2012). Members of the Homer family of adaptor proteins that can anchor binding partners to the postsynaptic density (PSD) interact with a defined motif within the tail, and DAGLα can be co-immunoprecipitated with Homer 2 in Neuro-2a cells (Jung et al., 2007). DAGLα also co-immunoprecipitates with the calcium/calmodulin-dependent protein kinase II (CaMKII) via an interaction with the tail and this kinase is also enriched within the PSD (Shonesy et al., 2013). Thus a number of mechanisms are emerging that might to some extent govern the molecular interactions of DAGLα at the PSD, but these do not speak to the dynamics of DAGLα trafficking to and within dendritic spines.

The dendritic spine has long been recognized as a highly dynamic structure, with changes in both spine morphology and molecular composition of the postsynaptic membrane impacting on synaptic plasticity (Anggono and Huganir, 2012; Hoogenraad and Bradke, 2009). The location of DAGLα within the dendrites and cells will determine not only where 2-AG can be made, but might also govern how 2-AG is released. With this in mind we embarked on a study to determine to what extent DAGLα exists in a dynamic as opposed to a static pool at the cell surface in dendrites and in cells. Our results have revealed for the first time that DAGLα can cycle within endosomes between an intracellular pool and a cell surface pool in both neuronal dendrites and COS-7 cells, the latter in a PKC-dependent manner. This newly discovered dynamic trafficking of DAGLα points to new sites of 2-AG synthesis and provides for a mechanism to regulate surface levels of the enzyme. Both are likely to impact on eCB signaling.

Results

Design and verification of the epitope-tagged DAGLα constructs

The DAGLα protein consists of a short intracellular N-terminal (22 amino acids) and four transmembrane domains (green), followed by a catalytic domain (pink) and a relatively long (~370 amino acids) tail at the C-terminus (shown schematically in Figure 1A). The extracellular loops are poorly conserved and have no obvious binding function (Reisenberg et al., 2012). We therefore designed a construct expressing human DAGLα with a HA tag inserted in the first extracellular loop, retaining an intracellular V5 tag located on the C-terminal of a previously described DAGLα construct (Bisogno et al., 2003). The correct insertion of the HA sequence was confirmed by sequencing and Western blot; furthermore, immunostaining analysis of a number of cell types showed the construct to be expressed in a manner that was not obviously different from constructs lacking the extracellular tag. The specificity of staining the V5 tag was proved by expressing the construct in COS-7 cells; here V5 antibody staining was not detected in untransfected cells and could only be detected in cells that express the transgene once they had been permeabilised (data not shown). The HA-DAGLα construct was then transfected into cultured hippocampal neurons and the expression detected on the cell surface of live cells with an anti-HA antibody two days after transfection. The distribution of cell surface DAGLα pool was similar to the total DAGLα distribution as revealed by the V5 tag labeling, exhibiting a clear punctate pattern in the dendrites (Figure 1B, C), with extensive overlap confirmed by colocalization analysis (Fig 1D). However, importantly, some V5-positive puncta were not labeled with HA, suggesting that DAGLα may be present in intracellular compartments (arrowheads in Figure 1C).

To reveal the synaptic localization of surface DAGLα, we triple labeled the transfected neurons with the above tags and an antibody to Homer 1, a canonical PSD marker (Shiraishi-Yamaguchi and Furuichi, 2007). There was extensive co-

localization between DAGLα on the dendritic surface and Homer (Figure 1B, C, D). These results show that the surface pool of DAGLα in the dendrites of hippocampal neurons is located either at or in near vicinity of the PSD, mirroring the presynaptic distribution of the surface CB1 (Dudok et al., 2014).

DAGLα is constitutively internalized and recycled in hippocampal neurons

We next tested the fate of the cell surface pool of DAGLα. To this end, hippocampal neurons transfected with HA-DAGLα were live-labeled for HA and allowed to internalize the label for 20 min. The cultures were then fixed and stained for the antibody/DAGLα complex at the cell surface and then permeabilized to stain for any internalized antibody/DAGLα complex (see Methods for details). As before, two pools of DAGLα were detected, a surface pool and an internalized pool in the cell body as well as in the neuronal dendrites (arrowheads in Figure 2A).

In order to follow the fate of the internalized DAGLα, a recycling assay was carried out (see Methods for details). Briefly, cells were allowed to internalize the label as above and then subjected to an “acid wash” to remove surface-bound antibody whilst leaving the internalized antibody-receptor complexes intact (Figure 2B). If the internalized antibody/DAGLα complex is retained within the cell, the antibody will not reappear on the cell surface. In contrast, the reappearance of the label at the cell surface would indicate recycling back to the cell surface. After 30 min, the HA label was again readily detected at the cell surface (Figure 2C). Thus, DAGLα is constitutively internalized and recycled back to the cell surface in neurons.

DAGLα is constitutively internalized and recycled in COS-7 cells

For further study of DAGLα trafficking, we turned to COS-7 cells as a model system

due to their ease of culturing and morphology favoring imaging studies. DAGLα on the surface of COS-7 cells was labeled in live cell cultures as before and returned to the incubator at 37°C for various times to allow endocytosis. As in neurons, internalization of surface DAGLα resulted in its accumulation in intracellular punctate structures, indicating that DAGLα is constitutively internalized in the COS-7 cells (Figure 3A). Internalization was evident after 20 min and increased over time at a steady rate (Figure 3B).

The next step was to test if antibody/DAGLα complex can be recycled back to the cell surface. Cells expressing the DAGLα construct were live-labeled and then either washed and fixed directly or acid washed before fixation (Figure 4A, B). The acid wash step again completely stripped the HA-antibody from the cell surface (compare Figure 4A and Figure 4B). As expected, internalized antibody/DAGLα complexes can readily be detected after incubating the cells at 37°C for 30 min and acid wash to strip the surface bound antibodies provided the cells are permeabilized (Figure 4C). The next step was to test if antibody/DAGLα complex can be recycled back to the cell surface. Cells that had been antibody fed and incubated for 30 min at 37°C were acid washed and returned to the incubator for another 30 min. At this time point antibody/DAGLα complexes were again readily detected at the cell surface (Figure 4D). Therefore, as with neuronal dendrites, DAGLα can be internalized and recycled back to the cell surface in COS-7 cells.

Internalized DAGLα co-localizes with microtubules but not F-actin

Based on cell staining it is apparent that the cell surface pool of DAGLα, as well as the intracellular pool, can be seen in highly organized domains. Depending on the plane of focus the staining is either highly punctuate, and/or tubular suggesting localization within a reticular structure (e.g. see Figure 3A). Based on this observation, we looked for co-localization of DAGLα with cytoskeleton markers

(phalloidin for F-actin, and antibodies against β-tubulin for microtubules). Confocal images revealed extensive co-localization between DAGLα and β-tubulin close to the plasma membrane (Figure 5A), however, co-localization with F-actin was very limited and/or less obvious (Figure 5B). Analysis of the images confirmed that the correlation of the intensities (shown as Pearson's R value) of DAGLα and β-Tubulin was significantly higher than the value from DAGLα and F-actin (Figure 5C).

Internalized DAGLα was transported to EEA1 and Rab5-positive early endosomes via clathrin-independent endocytic pathway

To investigate the intracellular itinerary of the internalized DAGLα, we first sought to identify the endocytic pathway involved in its internalization. Clathrin-mediated endocytosis (CME) represents the best characterized pathway for internalizing membrane glycoproteins, but alternative clathrin-independent pathways may also be involved (Mayor and Pagano, 2007). The rapidly internalized (5 min) pool of DAGLα did not co-localize with the clathrin labeled vesicular structures (Figure 6A) or a canonical CME cargo transferrin labeled with Alexa Fluor 555 (Tf-555) (data not shown), suggesting that endocytosis of DAGLα proceeded independent of formation of clathrin vesicles. However, a modest degree of co-localization between large DAGLα-positive puncta and clathrin was observed after 15 min endocytosis, consistent with a previously reported accumulation of clathrin in endosomal structures (Raiborg et al., 2001) (Figure 6B). These results indicate that DAGLα might be internalized via a clathrin-independent endocytic pathway (CIE), but sorted with clathrin-positive endosomes at a later stage. In agreement with this, internalized DAGLα strongly co-localized with canonical early endosome markers EEA1 (Figure 6C) and Rab5 (data not shown) (Nielsen et al., 1999). In contrast, there was no obvious co-localization between DAGLα and a lysosome marker LAMP1 (Figure 6D). Moreover, pharmacological inhibition of CME by using Dynasore, a potent Dynamin

inhibitor (Macia et al., 2006) at 100μM significantly inhibited the endocytosis of Tf-555, but DAGLα endocytosis was not significantly inhibited (Figure 6E). We therefore conclude that prior to reappearing on the cell surface DAGLα is transported to early endosomes most likely via a clathrin-independent pathway. Importantly, evidence suggests that the enzyme is not targeted to lysosomes.

DAGLα endocytosis is PKC-dependent

Having established that DAGLα can exist in two pools, we conducted some experiments to determine if cycling between them might be regulated by calcium and/or phosphorylation. As yet we have not seen any effect of treating cells with a calcium ionophore on the rate of DAGLα internalization (data not shown). However, treating the cells with a PKC inhibitor, Go 6976 (5 μM), significantly reduced the endocytosis of DAGLα by ~25% as compared to DMSO-treated controls (Figure 7B, D); note the number of DAGLα positive intracellular vesicles was also clearly reduced (Figure 7B). In contrast, treating cells with two selective PKA inhibitors (KT 5720 and H89, all used at 5 μM) had no significant effect on DAGLα internalization rate (Figure 7C, D and data not shown). Thus our data suggest DAGLα endocytosis is specifically regulated by PKC, but not PKA activity.

Discussion

The localization of DAGLα within cells will not only determine where 2-AG is made, but perhaps also impact on how it might be released. In this study, we provide much-needed tools allowing for detailed description of DAGLα localization and trafficking in neuronal and non-neuronal cells. The first important result is the observation that HA-DAGLα behaves like the endogenous enzyme in developed neurons in that it correctly targets to cell surface of dendritic spines as evidenced by co-localization with Homer 1 following live cell staining. However, a second dendritic pool of DAGLα

that could not be accessed by the HA-antibody in non-permeabilized cells was also found. This was closely associated with, but clearly distinct from, the cell surface pool. Thus, even in the absence of any perturbation, a cell surface and an intracellular pool of DAGLα can be identified in dendrites. In this study we have focused on one simple, but fundamental question. This is whether DAGLα can move from the surface pool to an intracellular pool and from there back to the cell surface. In this context, the trafficking of glutamate and other receptors by the recycling endosomes regulates synaptic plasticity by controlling receptor level and distribution at the membrane (Anggono and Huganir, 2012; Groc and Choquet, 2006).

Classic antibody feeding assays with live cells allow the fate of a labeled pool of cell surface receptors to be followed. In the present study this approach has led to results that can be summarized as follows. (1) The DAGLα on the cell surface at the PSD can be readily labeled with the HA-antibody and the complex is internalized into the dendrite. (2) The antibody/DAGLα complex is returned to the cell surface of the dendrite rather than being targeted for degradation. (3) The same phenomenon can be seen and better studied in COS-7 cells, where the complex went to the early endosome (as marked by EEA1 and Rab5), avoided the lysosome, and was returned to the cell surface. (4) DAGLα cycling can be regulated by intracellular signaling pathways. More specifically, endocytosis was significantly reduced as a consequence of treating cells with a PKC inhibitor. Although future studies will look at this in more detail, it is worth noting that other treatments including PKA inhibitors did not significantly affect the rate of DAGLα endocytosis.

Endocytosis is a process involving many molecules and signaling pathways. Endocytic pathways are generally divided into clathrin-mediated and clathrin-independent endocytosis (CME and CIE). CME is the most extensively studied endocytic pathway and transferrin is a glycoprotein constitutively internalized via

CME. At the initial stages of endocytosis DAGLα did not co-localize with clathrin or the newly internalized transferrin, however some overlap becomes apparent at a later stage. This is similar to the trafficking of AMPA receptors as previously reported (Glebov et al., 2015). Moreover, pharmacological inhibition of clathrin-dependent endocytosis by using a potent CME inhibitor, Dynasore does not inhibit DAGL endocytosis. Thus, DAGLα joins the growing list of neuronal cargos internalized through CIE.

Within the cell DAGLα localizes with EEA1 and Rab5-positive early endosomes, a common sorting station which has regions of thin tubular extensions and large vesicles that are located toward the cell periphery (Jovic et al, 2010). Here protein cargos can be sorted to late endosomes (which can mature into lysosomes where the protein is degraded) or recycling endosomes where the proteins are recycled back to plasma membrane (Arancibia-Carcamo et al, 2006). In this context, internalized DAGLα does not co-localize with lysosome markers and it is clearly recycled to the cell surface. It is also becoming clear that endocytosis into tubular compartments is a common event in mammalian cells (Doherty and McMahon, 2009). In this context, our results clearly show that DAGLα is associated with tubular structures in the cell. There was also clear co-localization of DAGLα with β-tubulin. It is well established that all types of endosome move actively along microtubules, and this is particularly clear in neurons (Granger et al., 2014). All of the above results support the hypothesis that DAGLα can be trafficked to and from the cell surface within endosomes mostly likely driven by microtubule motors. Importantly, following the development of the axon initial segment, early endosomes readily traffic into dendrites but are excluded from axons (Edwards et al., 2013; Rolls and Jegla, 2015); this provides a possible explanation for the polarized distribution of DAGLα in developed neurons.

2-AG synthesized by DAGLα at the cell surface is thought to accumulate in the membrane and get released from the cell based on a diffusion gradient, however there is much that we do not know (Alger, 2012). If 2-AG is synthesized by the DAGLα that is associated with tubular and/or vesicular endosomes it might stay associated with these structures and be released, like other lipids, associated with extracellular vesicles including exosomes (Raposo and Stoorvogel, 2013). Interestingly, exosomes secreted from microglia can carry active anandamide (Gabrielli et al., 2015), and DAGLβ protein and mRNA have recently been shown to be a component of exosomes released from endothelial cells (de Jong et al., 2012).

The above scheme provides a mechanism that could modulate eCB signaling by regulating the level of DAGLα at the cell surface and points to the possible synthesis of 2-AG at intracellular sites. The latter might help explain some observations that suggest the decoupling of release from synthesis. In this context synaptic activity is required to release pre-loaded eCBs from terminals (Adermark and Lovinger, 2007). In a similar vein, a use-dependent inhibition of DSE by DAGL inhibitors also suggests a decoupling of release from synthesis (Zhang et al., 2011). The observation that 2-AG mobilization requires an increase in calcium, in addition to an increase in substrate, would also be in keeping with this model (Shonesy et al., 2015). Interestingly, differences in the sensitivity of DSE and DSI to DAGL inhibitors can be found in the same terminal following both external and internal application of the drugs (Zhang et al., 2011) leading the authors to propose physically and/or functionally distinct DAGLα pools in dendrites. It would be interesting to see how neuronal activity affects the localization of the DAGLα in the context of activity-dependent dynamics of synaptic geometry (Glebov et al., 2016).

In summary, this is the first description of DAGLα cycling between the cell surface and an intracellular endosomal compartment. DAGLα cycling is shown to be a

regulated process pointing to a new mechanism that can regulate the level of the enzyme at the cell surface, and points to possible new sites of 2-AG synthesis and release mechanisms from cells.

Materials and methods

Cell cultures

Dissociated hippocampal cultures were prepared from embryonic day 18 Sprague Dawley rats as previously described (Kaech and Banker, 2006). Neurons were dissociated using 0.05% trypsin-EDTA followed by washing and trituration and then plated onto 13 mm diameter glass coverslips coated with poly-L-lysine in 4-well dishes. All cell lines were grown in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin and GlutaMAX (all Life Technologies, UK). All cultures were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell transfection

Hippocampal neurons were transfected on 10-14 days in vitro (DIV) using the NeuroMag Magnetofection system according to manufacturer's instruction (OZ Biosciences, San Diego, USA). In brief, indicated quantity of DNA was diluted in culture medium without serum and supplement. The DNA solution was added to the NeuroMag solutions by vigorous pipetting or brief vortexing and incubated at room temperature for 15 to 20 min. The NeuroMag/DNA complexes were added onto cells drop by drop and gently rocking the plate to ensure a uniform distribution. The cell culture plate was then placed on the magnetic plate for 15 min.

Transfections of COS-7 cells were carried out using the FuGene HD transfection reagent (Promega, UK) in 6-well dishes according to the FuGene HD protocol database. In brief, cells growing in DMEM supplemented with 10% bovine serum were plated the day before transfection at appropriate density, DNA was added into

OPTI-MEM media (Life Technology, UK) and then FuGene HD reagent was added. This was mixed carefully by vortexing briefly and incubating for 10-15 min at room temperature before adding to the cells. The cells were cultured overnight and were generally trypsinised and seeded onto 13 mm diameter glass coverslips. Transgene expression was evaluated after 24 h.

Reagents and DNA plasmids

Rat or mouse anti-HA antibodies were from Roche (Roche, Switzerland); Rabbit anti-HA, anti-V5, anti-clathrin, anti-early endosome antigen 1 (EEA1) and anti-lysosomal-associated membrane protein 1 (LAMP1) antibodies were from Abcam (Abcam, UK); Rabbit anti-Homer was from Synaptic Systems (Synaptic Systems, Goettingen, Germany); Rabbit anti-Rab5 antibody was from Cell Signaling (Massachusetts, USA). Alexa Flour conjugated phalloidin, transferrin and secondary antibodies were from Life Technologies. PKC inhibitor Go 6976, PKA inhibitors H89 and KT 5720 were from Sigma (Sigma-Aldrich, UK). For HA-DAGLα construct, a HA peptide sequence (YPYDVPDYA) flanked by a glycine-serine-glycine-serine linker on either side was inserted into the first extracellular loop of Human DAGLα sequence in pcDNA™6.2-DEST/V5 construct (performed by GenScript, Piscataway, USA).

Antibody feeding and internalization assay

Antibody feeding and internalization assay were performed as previously described (Arancibia-Carcamo et al., 2006; Glebov et al., 2015). In brief, after transfection of cultured hippocampal neurons or COS-7 cells with the HA-DAGLα constructs, an antibody against the extracellular epitope tag was added into the medium (culture medium supplemented with 25 mM HEPES and 0.5% BSA, pH 7.4) of live cells for 20 min at room temperature for neurons or 4°C for COS-7 cells. The cells were then gently washed with PBS to remove unbound antibody and either fixed for a zero-time point control or returned to the incubator for various times for internalization. Cells

were then briefly fixed with 4% (w/v) paraformaldehyde (PFA) and a fluorophore conjugated secondary antibody was used to label the antibodies that associate with the surface pool of the target protein. Cells were then permeabilized with 0.2% (v/v) Triton X-100 and subsequently another fluorophore conjugated secondary antibody was used to label the extracellular tag associated with the internalized pool of the target protein. Coverslips were then mounted for imaging.

Recycling/acid wash assay

An acid wash assay was performed as previously described (Arancibia-Carcamo et al., 2006). In brief, cultured hippocampal neurons or COS-7 cells expressing HA-DAGLα were live labeled as stated above. The cells were either washed and fixed directly for a control or washed in a DMEM acid buffer (pH 3.0-3.5) before fixation to check acid wash efficiency. An anti-rat 488 secondary antibody was then used to detect the anti-HA antibody on the cell surface. Another group of cells were live labeled and PBS washed and returned back to the incubator for uptake for 30 min. After incubation, the cells were washed in acid buffer and either fixed (to test if the internalized antibody-enzyme complexes could still be detected after this acid wash step) or returned back to the incubator for another 30 min for the internalized antibody-enzyme complexes to recycle before fixation. An anti-rat Alexa Fluor 488 secondary antibody was used to detect the anti-HA antibodies that remained on the cell surface. After permeabilization, an anti-rat Alexa Fluor 594 secondary antibody was used to detect the anti-HA antibodies associated with the internalized HA-DAGLα. Coverslips were subsequently mounted for imaging.

Image Analysis

Confocal images were taken by Zeiss LSM710 confocal microscope by collecting random fields from various regions of the coverslip. For quantification of internalization rates, imaging settings were kept consistent for all the time points for

each experiment. Images were analyzed by using ImageJ software. Only brightness/contrast was adjusted for visualization. Colocalization analysis was performed using a quantitative method (Costes et al., 2004) as previously described (Glebov et al., 2016). In brief, images were analyzed by using the ImageJ 'Coloc2' plugin with Costes automatic threshold regression to minimize the bias from threshold setting. Costes randomizations were set at 100. Only images with Costes P value > 0.95 were included in further analysis and the Pearson's correlation coefficient (Pearson's R value) was quantified. Internalization rate was calculated as previously described (Glebov et al., 2006; Glebov et al., 2015).

Statistical Analysis

Student's 2-tailed t-test and One-way or Two-way ANOVA followed by Bonferroni's post-tests were used for all statistical analysis. Where shown, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (all relative to control). For all graphs, the bars show mean values, while the error bars represent the standard error of the mean (SEM).

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Figure legends

Figure 1 Imaging cell surface and intracellular pools of DAGLα in hippocampal neurons.

(A), schematic of the labeling strategy. The DAGLα protein consists of an N-terminal and four transmembrane domains (green), followed by a catalytic domain (pink) and a C-terminal tail. To enable live cell labeling of DAGLα, an HA-DAGLα construct was made by inserting the HA peptide sequence (YPYDVPDYA) flanked by a glycine-serine-glycine-serine (GSGS) linker on either side into the first extracellular loop of human DAGLα, while retaining the intracellular V5 epitope tag. (B) Rat hippocampal neurons were transfected with HA-DAGLα at 10 DIV and were live labeled at 12 DIV. Surface DAGLα labeled by HA tag (shown in green) co-localizes with total DAGLα labeled by V5 tag (shown in red), manifesting a punctate pattern on dendrites. Cell also triple stained for Homer 1 (shown in cyan) and a merged image was shown. (C) Zoomed-in view of the white box in (B). Note the presence of intracellular pools of DAGLα labeled for V5 but not HA (arrowheads). Surface DAGLα co-localizes with Homer 1 in neuronal dendrites. (D) 2D intensity histograms of the dendrite in (C) generated by Image J Coloc 2 plugin with Costes automatic threshold. Pearson's R value and Costes P value are shown for each colocalization analysis. Scale bars: 10 μm and 1 μm for the zoom in.

Figure 2 DAGLα is constitutively internalized and recycled in rat hippocampal neurons

Cultured rat hippocampal neurons were transfected by HA-DAGLα at 10 DIV and 3 days later, cells were live labeled and internalized for 20 min. Neurons were then fixed and an anti-rat Alexa Fluor 488 antibody was used to detect HA tag associated with surface DAGLα (shown in green). Cells were then permeabilized and an anti-Alexa Fluor 594 antibody was used to stain the internalized DAGLα pool (shown in red). Right panel shows the zoomed views of the box area. White arrowheads indicate the internalized DAGLα at the dendrites (A). Another set of neurons were transfected and live labeled in the same way as the cells in (A). Cells were then incubated at 37°C for 30 min and acid washed to strip surface HA antibodies. Cells were then fixed (B) or further incubated at 37°C for 30 min to allow recycling (C). Neurons were then fixed and stained for surface and internalized DAGLα by using anti-HA 488 and anti-HA 594 respectively. Confocal images showing the surface (green stain) and internalized (red stain) HA-DAGLα. As shown in the zoomed image in (C), surface HA-DAGLα was again detected after antibody stripping and incubation, indicating that the DAGLα can be recycled back to the cell membrane after having been internalized. Scale bars: 20 μm and 5 μm.

Figure 3 Quantification of DAGLα internalization in COS-7 cells

COS-7 cells were transfected with the HA-DAGLα construct using the FuGene transfection kit. 24 h later, a rat anti-HA antibody was added to the live cells for 20 min at 4°C. Cells were washed and fixed to give a zero-time point, or washed and returned to 37°C for 10 to 60 min to allow endocytosis. After incubation, cells were briefly fixed and an anti-rat Alexa Flour 488 secondary antibody was used to detect the anti-HA antibody associated with DAGLα on the cell membrane. Cells were then permeabilized and an anti-rat Alexa Flour 594 antibody was used to label the anti-HA antibody associated with the internalized pool of DAGLα. Confocal images were taken under the same setting for all time points. Note the punctate red stained vesicles that accumulate inside of the cell after incubation (A). Quantification of DAGLα endocytosis in COS-7 cells is shown in (B). The green and red fluorophore intensities which were associated with surface and internalized DAGLα respectively were measured. The intensity ratio (red / green) that indicates the rate of internalization of DAGLα was calculated. The results show DAGLα was significantly internalized after 20 min incubation at 37°C, and the internalization increases with time to 60 min. Each bar represents the mean ± SEM (n=3, 10-15 fields were randomly taken from each coverslip, ~100 cells quantified for each group); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. One-way ANOVA followed by Bonferroni post-tests. Scale bar: 20 μm.

Figure 4 Internalized DAGLα is recycled back to the cell surface in COS-7 cells

COS-7 cells expressing HA-DAGLα were live labeled with the rat anti-HA tag antibody for 30 min at RT. The cells were either washed and fixed directly (A) or washed in a DMEM buffer (at pH 3.0-3.5) before fixation (B). An anti-rat Alexa Flour 488 secondary antibody was used to detect the anti-HA antibody on the cell surface (shown in green). Cells without the acid wash had extensive labeling for the anti-HA antibody on the cell surface (A). However, after acid wash, the surface staining was completely gone (B), indicating the success of antibody stripping. Another group of COS-7 cells were live labeled and washed as above and returned back to the incubator for antibody uptake for 30 min. After incubation, the cells were washed in acid buffer and either fixed (C) or returned back to the incubator for another 30 min for the internalized antibody-enzyme complexes to recycle before fixation (D). An anti-rat Alexa Fluor 488 secondary antibody was used to detect the anti-HA antibodies which remained on the cell surface. After permeabilization, an anti-rat Alexa Flour 594 secondary antibody was used to detect the anti-HA antibodies associated with the internalized HA-DAGLα. As shown by insert in (D), surface HA-DAGLα was again detected after antibody stripping and incubation, indicating that the DAGLα can be recycled back to the cell surface after having been internalized. Scale bar: 20 µm.

Figure 5 Internalized DAGLα co-localizes with β-Tubulin

COS-7 cells expressing HA-DAGLα were antibody fed with the rat anti-HA tag antibody for 20 min at 4°C and returned back to 37°C for 40 min. Cells were then acid washed to remove surface antibodies and fixed and permeabilized to stain for the internalized DAGLα. Cells were then incubated with an anti-β-Tubulin antibody to stain for microtubules or Alexa Flour 594 conjugated phalloidin was used to probe for F-actin (both shown in red). Confocal images showing the internalized DAGLα adjacent to plasma membrane co-localizes extensively with β-Tubulin (A). However, no obvious co-localization between DAGLα and F-actin was observed (B). Right panels of (A) and (B) are zoomed views of the rectangular boxes with the intensity profiles for the indicated dotted lines shown below. (C) Colocalization analysis between DAGLα and β-Tubulin or F-actin. Pearson's R value was calculated by using Image J Coloc 2 plugin with Costes automatic threshold without background subtraction. Shown are the mean values ± SEM (n = 4). Scale bar: 20 µm.

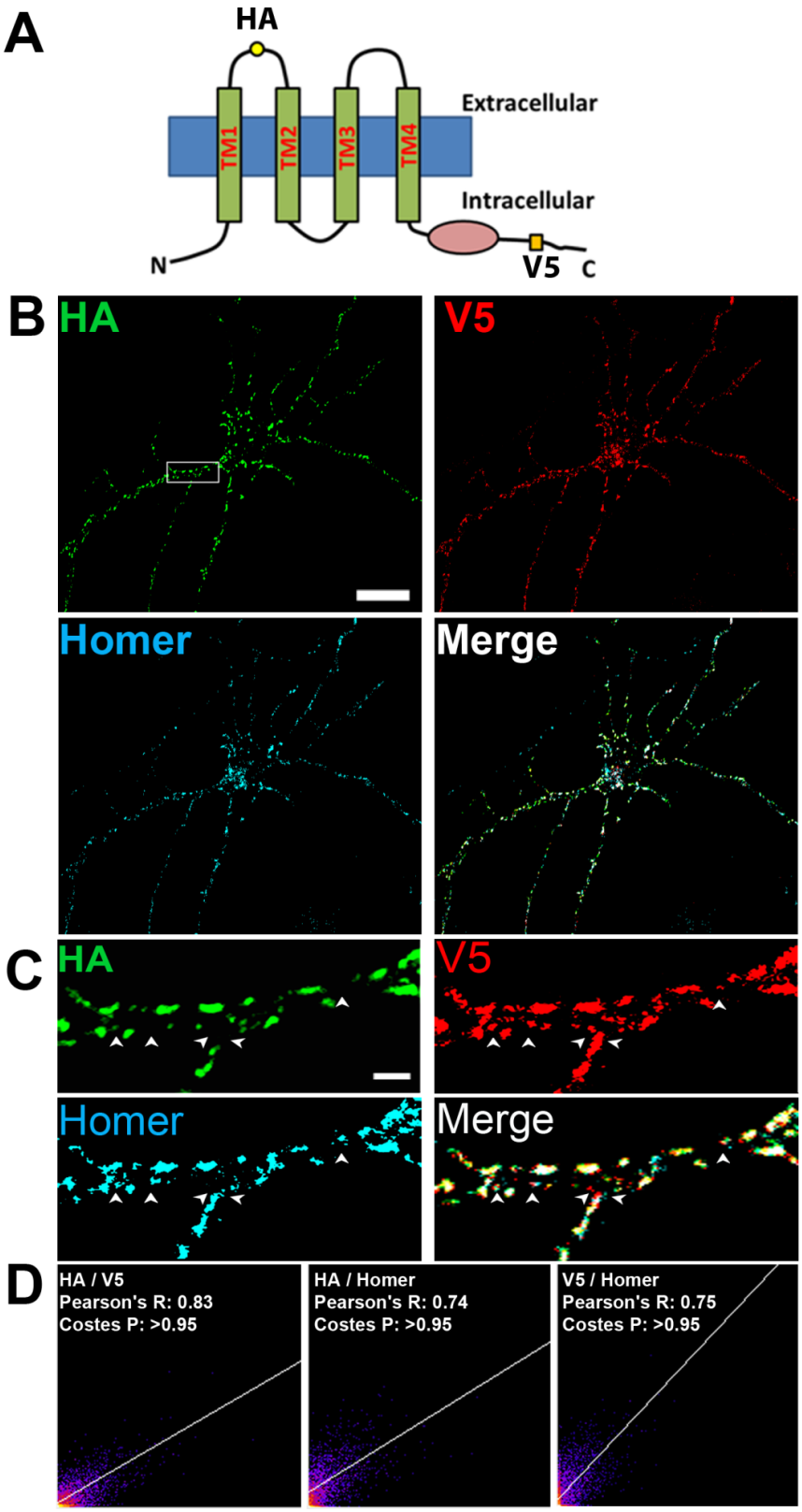
Figure 6 Internalized DAGLα was transported to early endosomes via clathrin-independent endocytosis in COS-7 cells

COS-7 cells expressing HA-DAGLα were live labeled as above and then returned back to 37°C for 5 (A) or 15 (B) min. Cells were then acid washed, fixed and permeabilized to stain for the internalized DAGLα (shown in green). Cells were also immunostained for Clathrin (shown in red). Another set of cells were treated the same as for A and B, but incubated for 20 or 40 min before fixation and immunostained for early endosome maker EEA1 and lysosome marker LAMP1, respectively. Right panels of each image are zoomed views of the rectangular boxes with the intensity profiles for the indicated dotted lines shown below. (E) Statistical analysis of internalization rate of Tf-555 and DAGLα treated with DMSO or a CME inhibitor Dynasore (100μM). Cells were internalized for 30 min. Incubation of cells with Dynasore significantly inhibit Tf-555 internalization, while the endocytosis of DAGLα was not significantly altered. Shown are the mean values ± SEM (n = 3 for Tf-555, 4 for DAGLα). Scale bar: 20 μm.

Figure 7 Endocytosis of DAGLα is inhibited by PKC inhibitor

COS-7 cells expressing HA-DAGLα were treated with Go 6976, KT5720 (both at 5 μM) or DMSO control for 10 min at 37°C. Cells were antibody fed with the rat anti-HA tag antibody for 20 min at 4°C and returned back to 37°C for 40 min in the presence of Go 6976, KT 5720 or DMSO before fixation. Cells were then fixed and anti-rat Alexa Fluor 488 antibody was used to stain the surface DAGLα (shown in green). Cells were then permeabilized to stain for the internalized DAGLα using another fluorophore conjugated secondary antibody (shown in red). Confocal images show that inhibiting PKC with Go 6976 significantly reduces the amount of DAGLα which appears in intracellular vesicles (compare A, B and C). (D) shows a quantification for DAGLα internalization rate at 40 min. Treatment with Go 6976 significantly inhibits DAGLα endocytosis. Shown are the mean values ± SEM (n = 3). Scale bar: 20 μm.

Fig 1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Extracellular
TM1
TM2
TM3
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Intracellular
N
V5
C
B
HA
V5
Homer
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V5
Homer
Merge
D
HA / V5
Pearson's R: 0.83
Costes P: >0.95
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V5 / Homer
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Costes P: >0.95

Fig 2

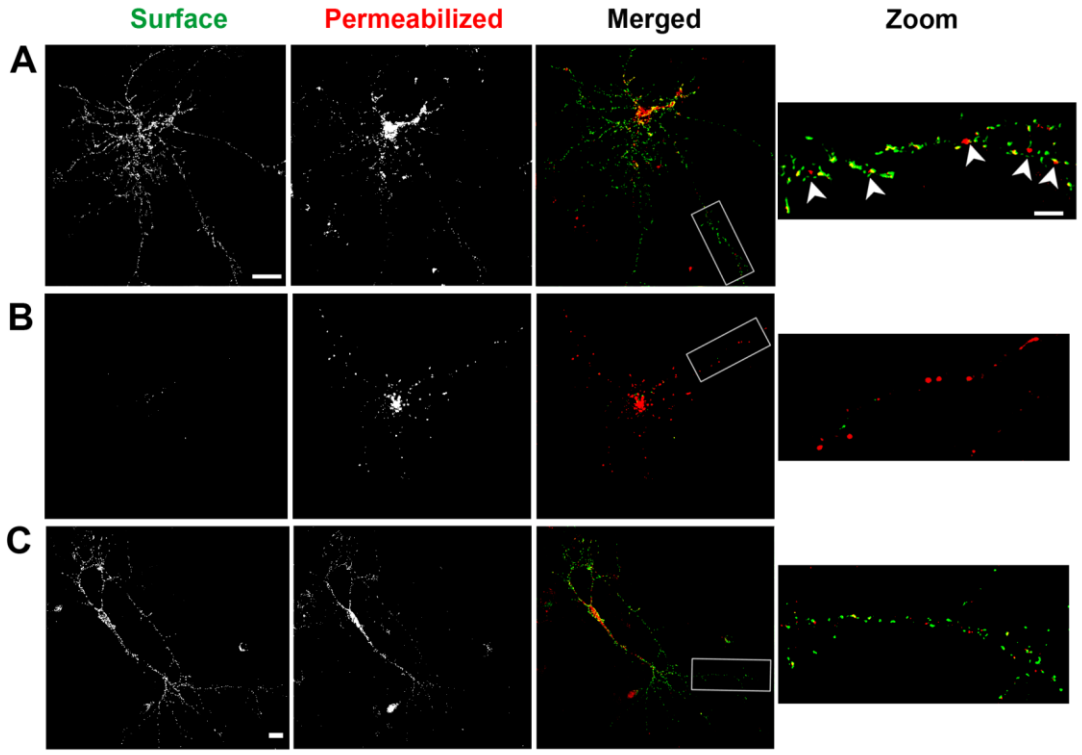
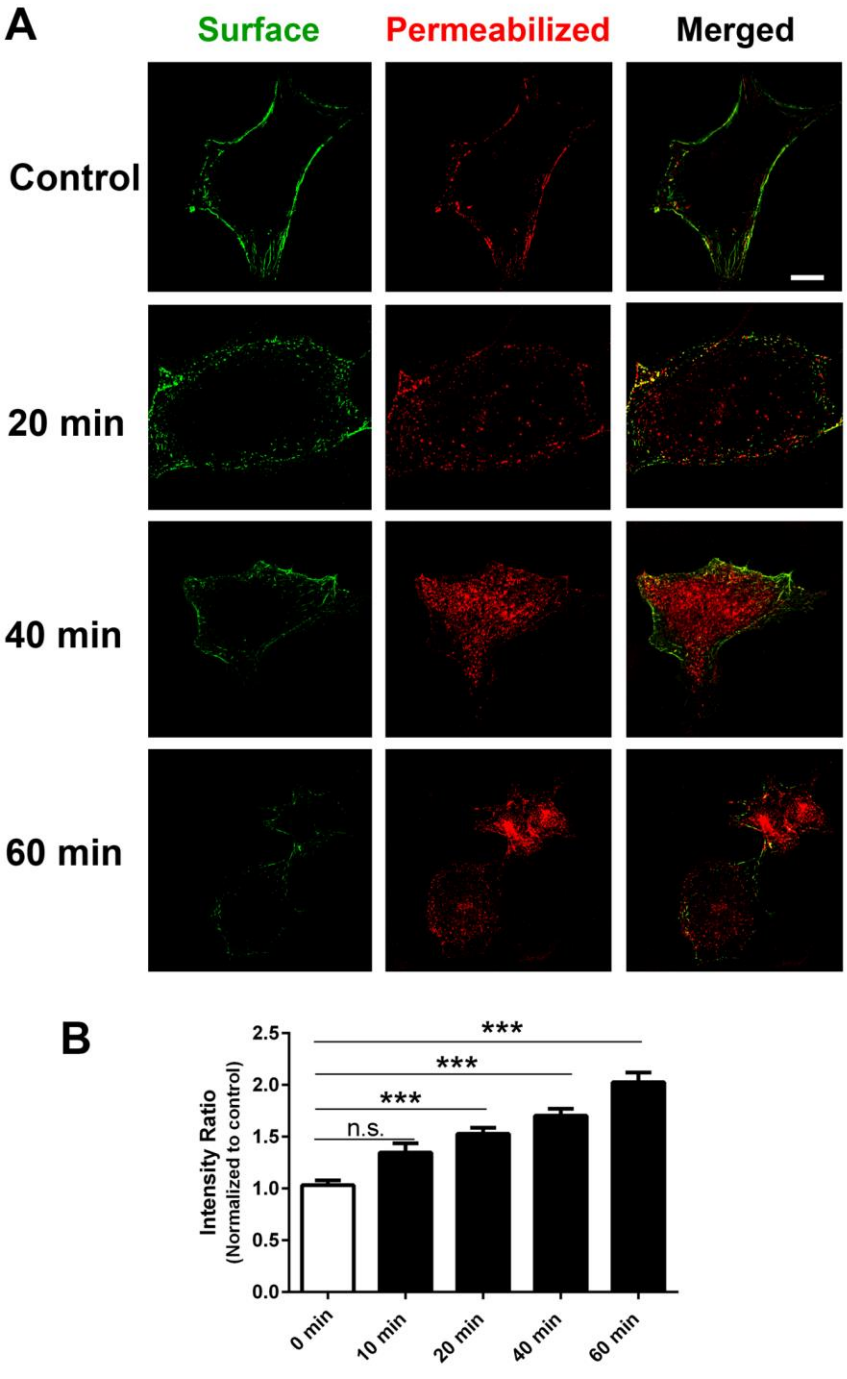


Fig 3


A
Surface
Permeabilized
Merged
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20 min
40 min
60 min
B
Intensity Ratio
(Normalized to control)
2.5
2.0
1.5
1.0
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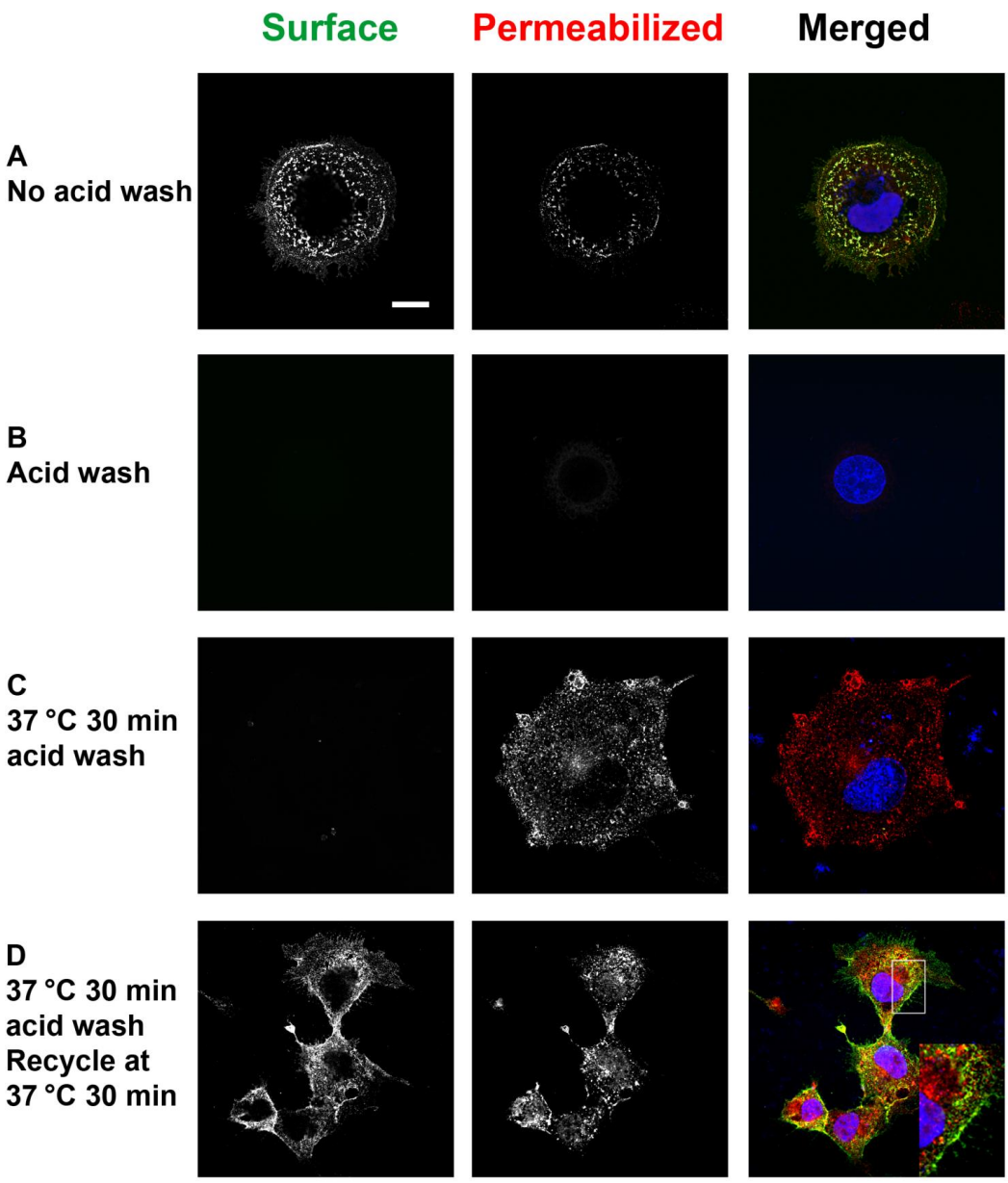
Fig 4


Surface
Permeabilized
Merged
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B
Acid wash
C
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acid wash
D
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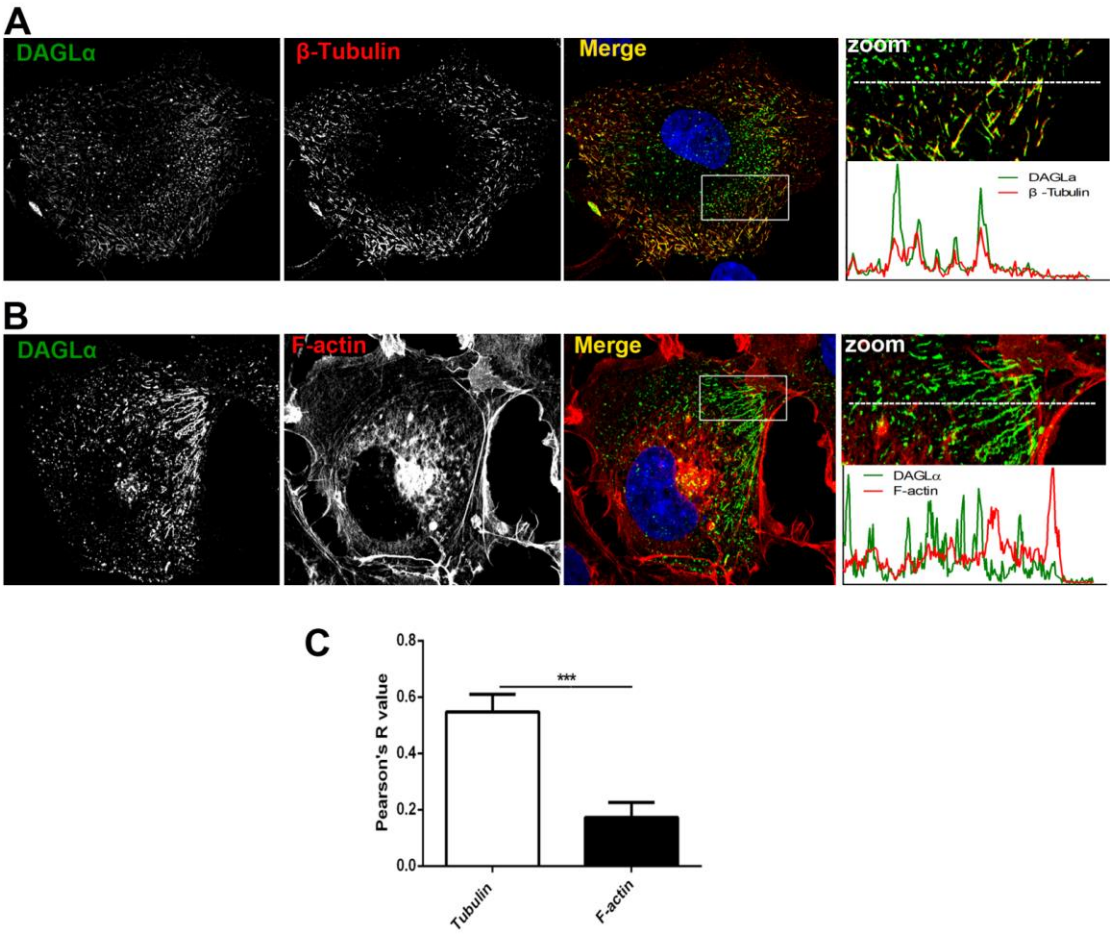
acid wash
Recycle at
37 °C 30 min

Fig 5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DAGLα
β-Tubulin
Merge
zoom
DAGLa
β -Tubulin
B
DAGLα
F-actin
Merge
zoom
DAGLα
F-actin
C
Pearson's R value
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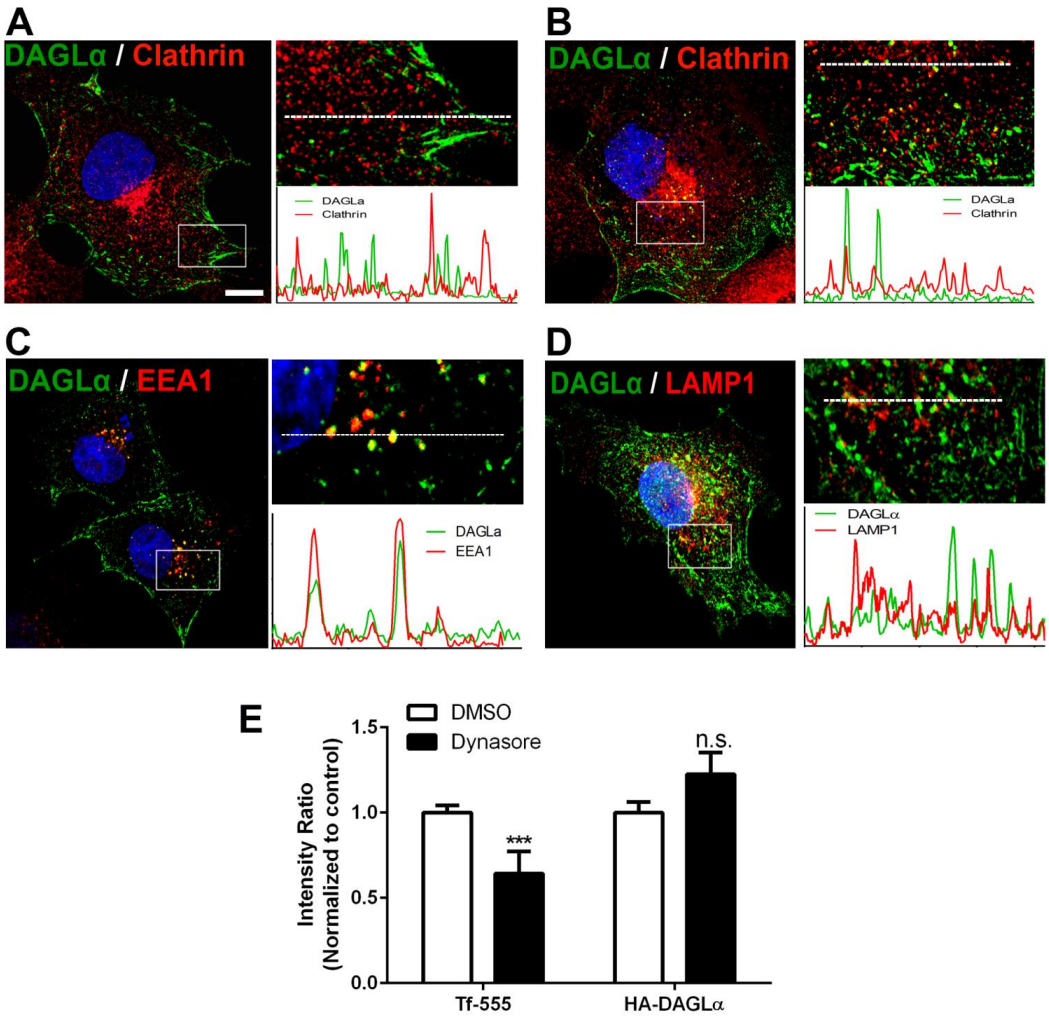
0.6
0.4
0.2
0.0
***
Tubulin
F-actin

Fig 6


A
DAGLα / Clathrin
DAGLa
Clathrin
B
DAGLα / Clathrin
DAGLa
Clathrin
C
DAGLα / EEA1
DAGLa
EEA1
D
DAGLα / LAMP1
DAGLα
LAMP1
E
DMSO
Dynasore
Intensity Ratio
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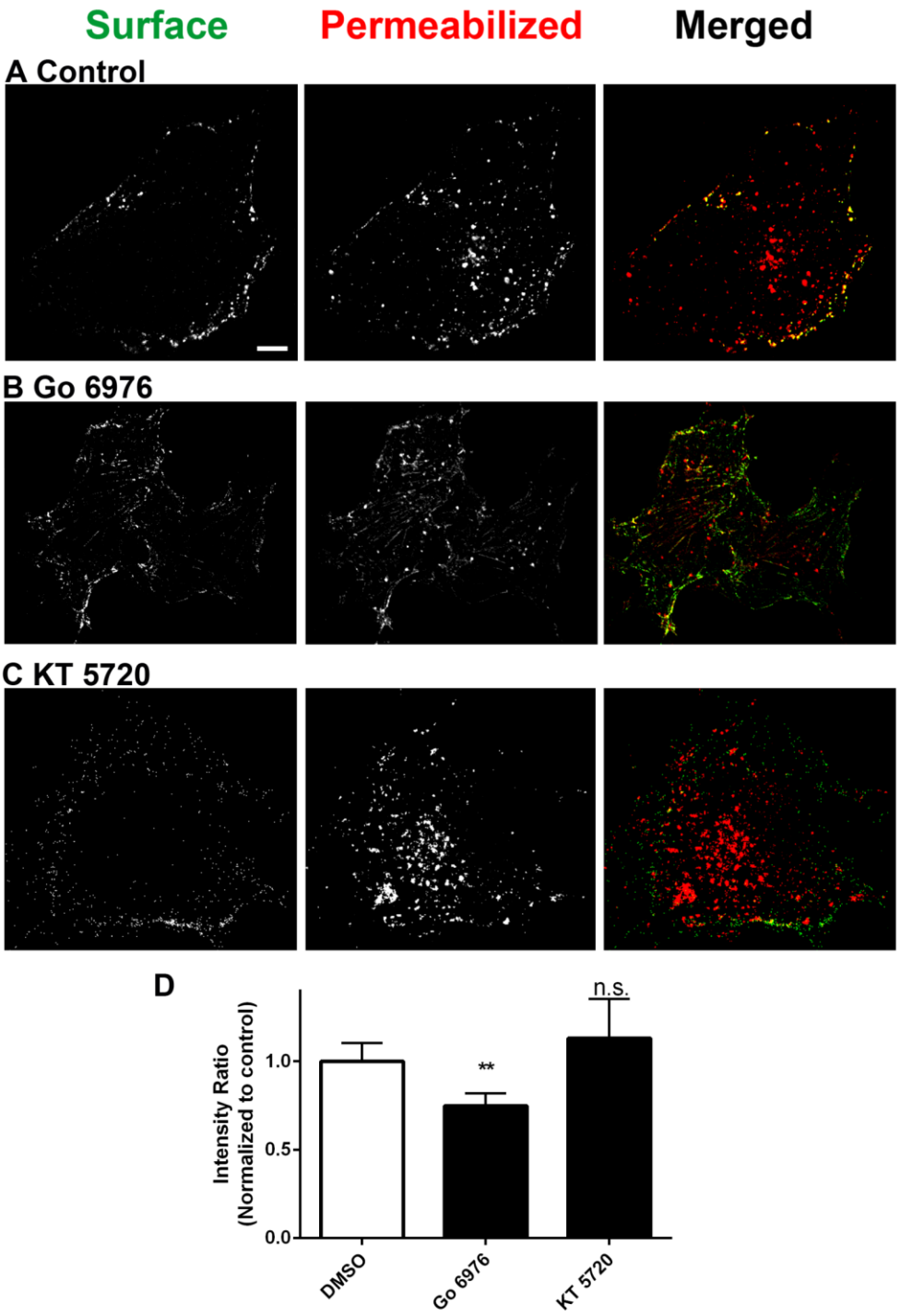
1.0
0.5
0.0
***
n.s.
Tf-555
HA-DAGLα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Permeabilized
Merged
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B Go 6976
C KT 5720
D
Intensity Ratio
(Normalized to control)
1.0
0.5
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n.s.
DMSO
Go 6976
KT 5720